0888-8809/02/$15.00/0
Printed in U.S.A.

Molecular Endocrinology 16(8):1778–1792

doi: 10.1210/me.2002-0089

EXHIBIT F

Identification of a Negative Regulatory Surface within Estrogen Receptor α Provides Evidence in Support of a Role for Corepressors in Regulating Cellular Responses to Agonists and Antagonists

HUEY-JING HUANG, JOHN D. NORRIS, AND DONALD P. McDONNELL

Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710

Several lines of evidence have indicated that the estrogen receptor (ER) can recruit the corepressors, nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid receptors (SMRT), to target genes in the presence of tamoxifen, suggesting a possible role for NCoR/SMRT in regulating ER pharmacology. However, a tamoxifen-dependent, direct interaction between NCoR/SMRT and ER *in vitro* has not been demonstrated. To investigate the possible involvement of different corepressors in the actions of antiestrogen-bound ER, we have constructed a phage display library that expresses 23-amino acid peptides containing the canonical CoRNR box motif in an otherwise random background. Screening of the CoRNR box library with apo-ER or ER treated with tamoxifen or ICI 182,780 led to the isolation of peptides whose ability to interact with ER was influenced by the nature of the bound ligand. Using a series of ERα mutants, we found that helix 12 was not required for the binding of CoRNR box peptides, whereas disruption of helixes 3 and 5 had a marked effect on peptide binding. One mutant, ER-L372R, lost the ability to interact with CoRNR box-containing peptides without affecting its binding to LXXLL motif-containing peptides. The estradiol- and tamoxifen-mediated transcriptional activity of ER-L372R was dramatically increased by 11- and 3-fold, respectively, compared with that of wild-type ERα. The ICI 182,780-mediated repressional activity of this mutant was also reduced by 4-fold compared with that of wild-type ERα. These results suggest that leucine 372 may be an important part of the interaction surface on ER that is responsible for corepressor binding. In addition, our data suggest that corepressors, other than NCoR/SMRT, may be involved in ER signaling. (*Molecular Endocrinology* 16: 1778–1792, 2002)

THE BIOLOGICAL actions of estrogens and antiestrogens are mediated by the estrogen receptor (ER), a hormone-activated transcription factor. Ligand-free (apo) ER can be isolated from cell extracts associated with complexes composed of a number of heat shock and immunophilin proteins (1, 2). The major nonreceptor component of these complexes is thought to be heat shock protein 90. However, there is evidence that repressor proteins other than heat shock protein 90 exist that interact with ER in the absence of hormone (3). Regardless, binding of hormone leads to dissociation of the apo-ER complex and subsequent receptor homodimerization. Depending on the nature of the bound ligand, the receptor can then interact with coactivators or corepressors and either positively or negatively regulate the transcription of estrogen-responsive genes. The complexity of this generalized model of estrogen action was increased with the identification of a second ER (ERβ) (4). Both ERα and ERβ bind estrogens with high affinity and can activate the transcription of promoters containing a classical estrogen response element (ERE) (5). However, ERβ has been shown to function as an inhibitor of ERα transcriptional activity, decreasing cellular sensitivity to estradiol in target cells where both receptor subtypes are expressed (6). Consequently, the response of a cell to specific ER modulators is determined to a large extent by the relative and absolute expression levels of coactivators, corepressors, and each receptor subtype.

Crystallographic analysis of the ER ligand binding domain (LBD) has established that ligand binding has a dramatic effect on receptor structure. Agonists such as 17β-estradiol and diethylstilbestrol induce a receptor conformation in which the carboxyl-terminal helix 12 (H12) is aligned over the hormone binding cavity, resulting in the formation of a specific binding site for

Abbreviations: DAX-1, Dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome; ER, estrogen receptor; ERE, estrogen response element; Gal4-DBD, Gal4 DNA binding domain; GRIP1, glucocorticoid receptor-interacting protein 1; H, helix; HBD, hormone binding domain; ID, receptor-interacting domain; LBD, ligand binding domain; NCoR, nuclear receptor corepressor; PPAR, peroxisome proliferator-activated receptor; PR, progesterone receptor; RAR, retinoic acid receptor; REA, repressor of estrogen receptor activity; RXR, retinoid X receptor; SERM, selective estrogen receptor modulator; SHP, short heterodimer partner; SMRT, silencing mediator of retinoid and thyroid receptors; SRC-1, steroid receptor coactivator 1; TR, thyroid hormone receptor; wt, wild-type.

EXHIBIT F

the consensus LXXLL motif found within coactivators (7, 8). Selective ER modulators (SERMs) such as raloxifene and tamoxifen, on the other hand, induce conformational changes in ER that interfere with the repositioning of H12 and alter the coactivator recruitment surface (7, 8). More recently, the structure of the ERβ LBD complexed with a pure antagonist, ICI 164,384, has been determined, indicating that binding of this antagonist completely destabilizes H12 and prevents it from adopting either agonist or SERM orientation (9). Proteolysis and peptide binding studies have also shown that pure antagonists facilitate a conformational change within ER that is unique and different from that induced by agonists and SERMs (10–12). Thus, SERMs and pure antagonists are mechanistically distinct classes of compounds that, not surprisingly, exhibit different pharmacological profiles *in vivo*. Of particular note in this regard are studies demonstrating that tamoxifen-resistant breast tumors are highly responsive to treatment with pure antiestrogens. An attractive model with which to explain this differential response is that the corepressor/coactivator ratio in breast tumors changes during the course of treatment, enabling tamoxifen to engage a suitable coactivator and to function as an agonist. However, as the pure antagonist-ERα complex adopts a different conformation, its cofactor preferences are different and are not compatible with transcriptional activation (13–15). Clearly, there is a need to identify and characterize the coactivators and corepressors that interact with ER and determine how they interface with the receptor.

Two corepressors, nuclear receptor corepressor (NCoR) and silencing mediator of retinoid and thyroid receptors (SMRT), have been implicated in mediating ER signaling. Specifically, it has been noted that 1) ERα-NCoR or ERα-SMRT complexes can be formed in MCF-7 cells upon treatment with tamoxifen (13, 16); 2) tamoxifen manifests considerable agonist activity in mouse embryo fibroblasts derived from NCoR knockout mice (17); and 3) down-regulation of NCoR was observed in tamoxifen-resistant MCF-7 xenografts in an animal model (13). Despite this compelling functional data, attempts to demonstrate the direct interaction between ER and NCoR/SMRT have revealed that ER can bind to these corepressors regardless of agonist or antagonist treatment *in vitro* (14, 18). This difficulty in linking NCoR/SMRT-mediated regulation of ER pharmacology to a tamoxifen-dependent, direct protein-protein interaction may suggest that 1) the actions of these corepressors are indirect and require another protein to enable complex formation; or 2) there exists as yet unidentified corepressors that interact directly with ER. To address these issues we have constructed a focused phage display library that contains the CoRNR box motif, derived by aligning two receptor-interacting domains (ID) from NCoR and SMRT. The CoRNR box motif has been shown to be important for binding to thyroid hormone receptor (TR) and retinoic acid receptor (RAR) (19, 20), and the sequences adjacent to the CoRNR box motif have been suggested to play an important role in mediating the specificity of receptor interactions (21). In this study we have used phage display technology to identify CoRNR box-containing peptides that bind ER specifically. Furthermore, disruption of the surfaces on ERα that facilitate CoRNR box peptide-receptor interactions have dramatic effects on ERα pharmacology, leading to the conclusion that this receptor is subject to negative regulation either by an inhibitory intramolecular interaction or by an as yet unidentified corepressor.

RESULTS

Characterization of the Phage Display Peptide Library

A 23-mer phage library in which the CoRNR box motif was flanked on each side by seven random amino acid residues (X_7-L-X-X-H/I-I-X-X-X-I/L-X_7) was created in the phage vector mBAX such that the recombinant peptide is expressed as a fusion with the pIII capsid protein in an M13 bacteriophage. Enrichment of the CoRNR box motif in the library was expected to facilitate the identification of NCoR/SMRT-like peptides that otherwise have a lower probability to be identified using a random peptide library. DNA from 36 random phage clones were isolated and sequenced, and the peptide sequences were deduced. Thirty-four clones expressed the expected 23-residue CoRNR box-containing peptide (Table 1). The overall amino acid distribution for the random codons is shown in Table 2A. To eliminate the bias inherent in codon usage, the frequency of each amino acid was divided by the number of different codons encoding that particular amino acid (Table 2, last column). Acidic amino acids such as aspartate and glutamate were the most abundant amino acids in this library (≥5%). Cysteine was very rare (0.3%). For the amino acid residue at position 4 in the peptide, an MWC codon (M = A or C; W = A or T) was used. This codon will encode not only histidine and isoleucine, which are found in the consensus CoRNR box sequence, but also asparagine and leucine. Table 2B shows that 29% of the peptides expressed the desired histidine/isoleucine. For the amino acid residue at position 9 in the peptide, an MTC codon was used, which only allows isoleucine and leucine to be expressed. The frequency of occurrence at this position is 56% and 44% for leucine and isoleucine, respectively, very close to the expected frequencies (Table 2B).

Affinity Selection of Peptides That Bind apo-ER or Complexes of ER with Different Antiestrogens

The CoRNR library was screened for phage-expressing peptides that bound with high affinity to ERα or ERβ in the absence of hormone or in the

EXHIBIT F

Table 1. Peptide Sequences of 34 Randomly Chosen Clones from CoRNR Box Library

Clone	H I xxxxxxxLxxIIxxxLxxxxxxx (N) (L)
1	ELITANPLWMNIASPLRVSLQKE
2	TWKPTTELGTIIGNRIMADRSSM
3	TGGRSAILADLIRADIATDRSKI
4	LREATQFLDANISQQIGFSWDPN
5	LEGWNKYLAHNIQRAIGGRASSL
6	YGFADGSLTQNIASTLSRAHSNF
7	MSQVRQDLSEHIENKLTENLVSR
8	SHSASYSLPTHILSSLGEMTMSK
9	HIYTSQELQEIIWDKLHPWSESP
10	SAGHVHPLGPNIKGSITPGFTAS
11	ETPNSSVLSVLIEQNISPSAMVR
12	GGYVAKQLSVNITSHLSQYKTEL
13	LHSPSEELHKNIHRTLKSAHTVW
14	YSDLSKSLQLHITRNIKGDSDKS
15	DTRLNTRLHPLINTSLLLSQSVP
16	PRSKLTILQSNIDQRLQRTVPGW
17	VPTTVPVLTTLISQGIDERRLLE
18	MFPRGNLLTNLIRPEIGLENRPY
19	CGSHDSTLCLNIPAQLLPVKQMT
20	DSKTSRSLNEHITFPIRPAGADS
21	TTVDGWSLRQNIPMRIKTQSTQS
22	MFVDDSVLGGNIGFQLVLAELQD
23	INRAVLDLDTNITTKLQHQRSHF
24	NEPHNVKLGWNIVSRLSMKVKRT
25	PVLPEMDLHENIHNLILIEQDHY
26	TDRANMLLPTHITTQLPDLAKRM
27	QSIMHEKLRWNIGEQLSLVERPS
28	FNSHPTELMRLIEQPITRYMMNE
29	QRGKLTMLEEIIINQIEDSVVST
30	QQQNRYLLAGLIRQTIIETPGNV
31	SQGVAAALKAHILIPLGQPRGMA
32	DPASAFGLQPLIHPRLMTQPSSL
33	YTSEVTALQSHIKATLRTPRKST
34	QETPHDRLWKNIRPTLSLFNKDM

Residues that were fixed to display the desired amino acids are in *bold*.

Table 2. Amino Acid Distribution of the CoRNR Box Library

−2 −1 1 2 3 4 5 6 7 8 9

H I

x x x x x x x L x x I I x x x L x x x x x x x

(N)

(L)

A) Random Codons

Amino Acid	Codons	Expected Frequency (%)[a]	Observed Frequency [% (no.)]	Observed Frequency/Amino Acid Redundancy (%)
Arg	CGK, AGG	9.4	7.4 (48/646)	2.5
Leu	CTK, TTG	9.4	5.4 (35/646)	1.8
Ser	TCK, AGT	9.4	2.2 (79/646)	4.1
Ala	GCK	6.2	5.9 (38/646)	3.0
Gly	GGK	6.2	5.7 (37/646)	2.9
Pro	CCK	6.2	7.0 (45/646)	3.5
Thr	ACK	6.2	9.6 (62/646)	4.8
Gln	CAG, TAG	6.2	7.0 (45/646)	3.5
Val	GTK	6.2	4.8 (31/646)	2.4
Asn	AAT	3.1	4.2 (27/646)	4.2
Asp	GAT	3.1	5.0 (32/646)	5.0
Cys	TGT	3.1	0.3 (2/646)	0.3
Glu	GAG	3.1	6.0 (39/646)	6.0
His	CAT	3.1	3.7 (24/646)	3.7
Ile	ATT	3.1	1.7 (11/646)	1.7
Lys	AAG	3.1	4.8 (31/646)	4.8
Met	ATG	3.1	3.6 (23/646)	3.6
Phe	TTT	3.1	2.0 (13/646)	2.0
Trp	TGG	3.1	1.9 (12/646)	1.9
Tyr	TAT	3.1	1.9 (12/646)	1.9

B) Specified Codons

Position	Amino Acid	Codons	Expected Frequency (%)	Observed Frequency [% (no.)]
4	His	CAC	25	21 (7/34)
	Ile	ATC	25	8 (3/34)
	Leu	CTC	25	24 (8/34)
	Asn	AAC	25	47 (16/34)
9	Leu	CTC	50	56 (19/34)
	Ile	ATC	50	44 (15/34)

[a] Expected frequency = no. of codons for that amino acid ÷ 32 codons × 100%. Note the use of reduced genetic code NNK (32 codons) in library construction; no. of sequenced codons = 646 (34 clones × 19 random codons).

presence of tamoxifen or ICI 182,780. After three rounds of affinity selection, significant enrichment of phage was observed using ERβ, whereas minimal enrichment of ERα interactors was observed. We believe that this is due to differences in the stability of the purified recombinant receptors, as most of the peptides obtained using ERβ as the target interact in an efficient manner with ERα in a mammalian two-hybrid assay (see below). Because of the difficulty of using ERα in the screen, we used ERβ as the target for all subsequent primary screens. ELISA was used to identify the phage clones that bind ER with high affinity and ligand dependency. Of 48 random clones analyzed in each pool, 32 clones bound apo-ERβ, 17 clones bound tamoxifen-ERβ, and 31 clones bound ICI 182,780-ERβ. The amino acid sequence of the interacting peptides was deduced after DNA sequencing of the phage inserts. Table 3 shows the peptide sequences and frequency of occurrence for the phage clones obtained. The bN, bT, and bI phage clones represent phage isolated using apo-ERβ, tamoxifen-ERβ, and ICI 182,780-ERβ as targets, respectively. All of the bT and bI phage were isolated using receptor immobilized directly to plastic plates. However, enrichment of bN phage can only be obtained using receptor bound to immobilized EREs, suggesting that apo-ER may adopt a more favorable conformation for CoRNR box pep-

EXHIBIT F

Table 3. Sequences of CoRNR Box-Containing Peptides that Interact with ER *in Vitro*

H I
xxxxxxxLxxIIxxxLxxxxxxx
(N)
(L)

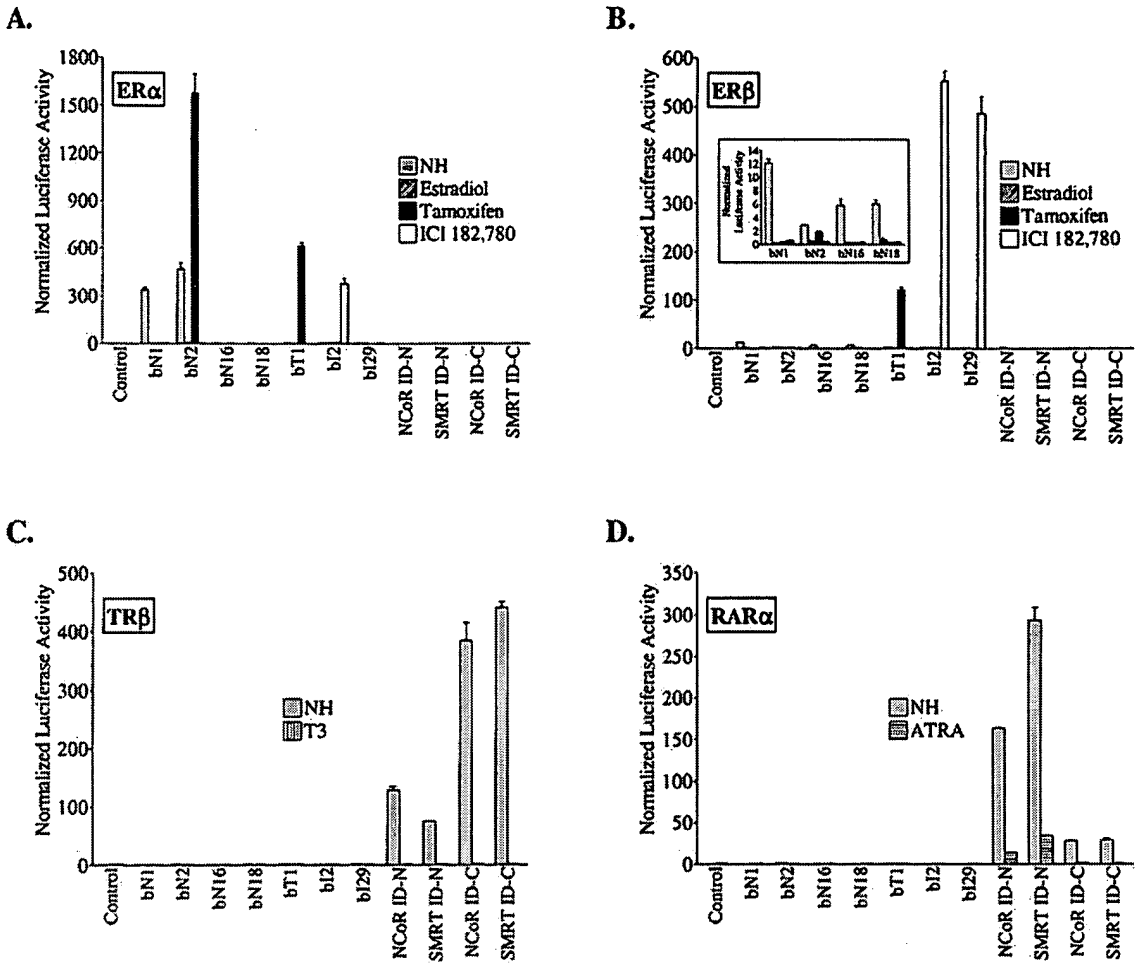
Peptide	Sequence	Frequency
bN1	QETIQRWLRGHIQRELGTMELKD	14/32
bN2	EYHEKRWLEGHIHHRIKSLLENS	16/32
bN16	HSTTLTGLASIIRERILTELRDE	1/32
bN18	PENFRQALRAHIADLITNQDYRS	1/32
bT1	ELFDAFQLRQLILRGLQDDIPYH	16/17
bT17 (=bN2)	EYHEKRWLEGHIHHRIKSLLENS	1/17
bI1	FMNNGVLLATNIENLLRTQPGSN	1/31
bI2	EMEWMKALRQHISGELRRNYTEE	5/31
bI5	NAAPRTALSHHIHSDLLDGPTTT	1/31
bI7	PKTPGVPLNPLISPEITSDTSML	1/31
bI8	AAYDPAALNNNIRYALVKHSQIK	3/31
bI9	NTYNTGALRFNIVESIWASKKLR	1/31
bI11	VPRFTKGLVGNIPLAIDTNSGTV	1/31
bI12	ESERANLLKEHIKMTLPEERKKT	1/31
bI13	NVIAQPTLASIIPPSLKRQSEAR	1/31
bI15	SPFTQVTLKGNIAPSIVGSQGMA	1/31
bI16	PIAHRVNLRHNITEDITLSHRFL	1/31
bI17	IDDGHPHLWKNIWDTLDKPGLGA	1/31
bI18	FKPGTSSLDTHIPLGLNKSFHHN	1/31
bI20	NQGNTKELLGNINYFLTHHTVPA	1/31
bI21	LTYPIRELKMNITSGIRLDKRVL	1/31
bI22	HKDSQLTLANNIMGQLSSTGGKH	1/31
bI23	WDVGEIRLRRHIKMPLSEEAIAE	1/31
bI24	SHYEKYSLPGIIVRKISTTDWRP	1/31
bI25	QHQNRQQLGSNIAATLPGKRESV	1/31
bI26	KSQTKAELPYLIGKQITKNQPEQ	1/31
bI27	DALDNQRLLGNIDNVLKVTNPNA	1/31
bI28	GQSARFALSQHIPSKIYDHPRPN	1/31
bI29	KWESLDALQGLISSHLSAMGPIP	2/31
bI30	RAVHERALNPLILDRLLHELKSE	1/31

bN peptides were isolated using ERβ immobilized on biotinylated ERE. bT peptides were isolated using ERβ treated with 4-hydroxytamoxifen (1 μM). bI peptides were isolated using ERβ treated with ICI 182,780 (1 μM). Residues that were fixed to display the desired amino acids are in *bold*.

tide binding when an ERE is present. Among the four unique bN clones obtained, clones bN1 and bN2 were found most frequently. It was noticed that bN1 and bN2 both possess arginine at position −2, tryptophan at position −1, and glycine at position 3, the significance of which remains to be determined. Seventeen clones showed specific binding to tamoxifen-bound ERβ. However, 16 of the clones in this group share the same sequence (bT1). Importantly, bT17 has the same sequence as bN2, suggesting that ER may adopt a similar conformation at the binding site of this peptide in the absence of hormone or in the presence of tamoxifen. Twenty-four unique clones were obtained that showed specific binding to ICI 182,780-bound ER. However, only two of the bI clones (bI2 and bI29) showed specific binding to ER using the mammalian two-hybrid assay (see below), suggesting that ICI 182,780 treatment *in vitro* may have forced ER to adopt a conformation that is not normally seen *in vivo*.

A mammalian two-hybrid assay was used to confirm that the CoRNR box-containing peptides identified *in vitro* interact with ER in the context of an intact cell. Specifically, the DNA inserts from each phage were cloned into a vector that enabled the peptide to be expressed as a Gal4 DNA binding domain (Gal4-DBD)-peptide fusion protein. The ability of the fusion proteins to interact with full-length VP16-ERα or VP16-ERβ occupied by different estrogens/antiestrogens in mammalian cells was determined by assaying the ability of the complex to activate a luciferase reporter construct under the control of five copies of a Gal4 upstream enhancer element (5×Gal4-TATA-Luc). As a point of reference we also produced Gal4-DBD-peptide fusions corresponding to the previously identified IDs from NCoR and SMRT. A direct comparison of the sequences of all the peptides used in this study is shown in Fig. 1E. The fusion proteins were shown to be expressed at similar levels by Western blot analysis (data not shown). Figure 1, A and B, shows the results from a mammalian two-hybrid assay confirming the ER-peptide interactions in HepG2 cells. Interestingly, although the CoRNR box peptides chosen for this analysis were isolated using ERβ as a target, most peptides interact with ERα also (Fig. 1A). Most importantly, all of these CoRNR box peptides interact with ERα or ERβ in the absence of hormone or in the presence of tamoxifen or ICI 182,780, and none interacts with estradiol-bound ER. These results strengthen our hypothesis that the CoRNR box peptides bind to surfaces on ER that are important for corepressor binding. The mammalian two-hybrid assay was further validated by demonstrating that the four CoRNR box-containing peptides derived from NCoR/SMRT IDs were capable of interacting with apo-TRβ or apo-RARα, and these interactions were disrupted upon addition of thyroid hormone or all-*trans*-retinoic acid (Fig. 1, C and D). However, neither TRβ nor RARα was able to interact with the ER-interacting CoRNR box peptides identified in our screens (Fig. 1, C and D). Furthermore, we were unable to demonstrate an interaction of ERα or ERβ with any of the previously defined CoRNR box peptides found in NCoR/SMRT (Fig. 1, A and B). Identical results were obtained using HeLa cells (data not shown). In general, the ER binding profiles of the CoRNR box peptides evaluated *in vivo* reflect the characteristics observed using *in vitro* ELISA and confirm that there are surfaces on ER that can accommodate these CoRNR box-containing peptides. These results are consistent with the presence of a specific corepressor binding surface(s) on ERα and ERβ. However, the ER-binding corepressors may be different from the corepressors (NCoR/SMRT) bound to TR or RAR.

EXHIBIT F



E.

CoRNR box	xxxxxxxLxxHIxxxIxxxxxxx
library	I L
peptide	(N)
sequence	(L)
bN1	QETIQRWLRGHIQRELGTMELKD
bN2	EYHEKRWLEGHIHHRIKSLLENS
bN16	HSTTLTGLASIIRERILTELRDE
bN18	PENFRQALRAHIADLITNQDYRS
bT1	ELFDAFQLRQLILRGLQDDIPYH
bI2	EMEWMKALRQHISGELRRNYTEE
bI29	KWESLDALQGLISSHLSAMGPIP
NCoR ID-N	RTHRLITLADHICQIITQDFARN
SMRT ID-N	GHQRVVTLAQHISEVITQDYTRH
NCoR ID-C	DPASNLGLEDIIRKALMGSFDDK
SMRT ID-C	HASTNMGLEAIIRKALMGKYDQW

Fig. 1. Evaluation of the Interaction between Receptor and CoRNR Box-Containing Peptides in Mammalian Cells

HepG2 cells were transiently transfected with expression vectors for the Gal4-DBD-peptide fusion protein, a Gal4-responsive luciferase reporter construct (5×Gal4-TATA-Luc), the β-galactosidase control plasmid, and VP16-ERα (A), VP16-ERβ (B), VP16-TRβ (C), or VP16-RARα (D). Transfection of Gal4-DBD alone is included as a control. After transfections, cells were treated with 100 nM estrogen or antiestrogens (A and B), 100 nM T_3 (C), or 100 nM all-*trans*-retinoic acid (D) for 24 h and assayed for luciferase and β-galactosidase activities. Data are presented as normalized luciferase activity, which was obtained by dividing luciferase activity by β-galactosidase activity. The *inset* in B magnifies the lower part of the luciferase activity to emphasize the hormone-specific interaction between ERβ and CoRNR box-containing peptides. E, Sequence comparison between ER-interacting CoRNR box peptides and the peptides derived from NCoR/SMRT IDs.

EXHIBIT F

Determination of the Region within ERα Required for CoRNR Box Peptide Binding

A series of mutants was generated to define the region(s) within ER required for CoRNR box peptide binding. We selected ERα for a more extensive analysis in this study because its pharmacology is more clearly defined. A schematic diagram of ERα and the mutants used in the current study is shown in Fig. 2. Mutations in helix 12 have been shown previously to decrease ERα transcriptional activity and abolish the interaction of the receptor with the coactivator glucocorticoid receptor-interacting protein 1 (GRIP1) (22) and with LXXLL motif-containing peptides (23). Mutations of lysine 362, leucine 372, and valine 376 were chosen because amino acid substitutions at equivalent positions in TRα or RXRα are known to decrease their ability to interact with NCoR/SMRT and to abrogate ligand-independent repression activity (20). All of these mutations were created in the background of a full-length VP16-ERα protein to enable evaluation of receptor-peptide interactions using a mammalian two-hybrid assay in HepG2 cells. The interactions of several different classes of LXXLL peptides with mutant ERs were also analyzed for comparison. Three LXXLL peptides D11, D47, and F6 with distinct receptor binding characteristics (23) were used. The Gal4-DBD-GRIP1 NR-box fusion contains the middle three copies of the LXXLL motif found in the coactivator GRIP1. As shown in Fig. 3, A–D, mutations in helix 12 did not affect the binding of the CoRNR box peptides to ERα, but actually enhanced the binding of bT1 and bI2 peptides to the receptor. The interaction of the CoRNR box peptides and the ERα helix 12 mutants was interesting in that the exquisite hormonal specificity observed on the wild-type ERα (wtERα) was not preserved (Fig. 3, A–C). This suggests that the positioning of helix 12, influenced by the nature of the ligand bound, is an important regulator of CoRNR box peptide binding. Mutations at the amino acid residues Lys^{362} and Val^{376} within ERα decreased its ability to interact with both the CoRNR box and LXXLL peptides, suggesting that the binding surfaces for these two classes of peptides are close or overlapping. The results obtained with ER-L372R are the most interesting, as this specific mutation totally abolished the interaction between ERα and two of the CoRNR box peptides (bT1 and bI2) while having no effect on binding of the LXXLL peptides to the receptor (Fig. 3). Additional LXXLL peptides (D2, D22, and SRC-1 NR-box) were also tested, and they all exhibit similar binding characteristics (data not shown). Thus, although the binding sites on ERα for the CoRNR box and LXXLL peptides are closely linked, they can be functionally separated.

Using a mutant in which the N-terminal 311 amino acids were deleted (ER-HBD), it was shown that the interaction between ERα and all the CoRNR box-containing peptides were severely impaired (Fig. 3, A–D). The interaction of ER-HBD with LXXLL motif-containing peptides was affected, but not as dramatically (Fig. 4, E–H). These results suggest that domains other than LBD may be involved in the formation of the CoRNR box peptide binding pocket in ERα.

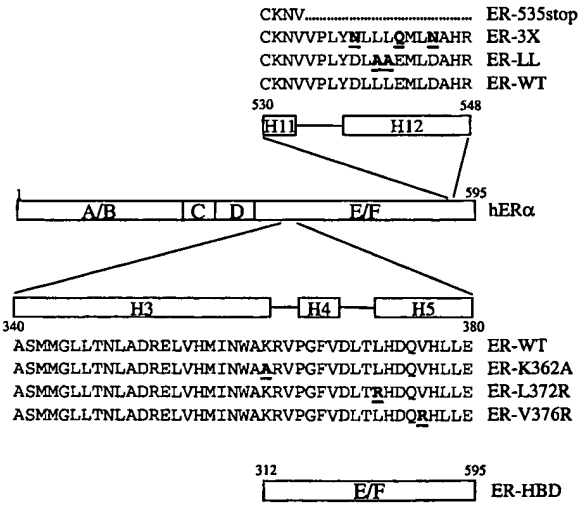


Fig. 2. ERα Mutants Used in This Study

Schematic representation of ERα showing amino acid substitutions in helixes H3, H5, and H12. Residues that were mutated are in *bold* and *underlined*. Also shown is a schematic of the ER mutant receptor (ER-HBD) that contains only the F domain and part of the E domain (H2 to H12).

Enhanced Transcriptional Activity by a Mutant ER That Is Unable to Bind to the CoRNR Box-Containing Peptides

The studies described to date indicate that the CoRNR box peptides identified can function as probes of antagonist-induced changes in ERα conformation. Next we determined whether the surfaces on the receptor with which these peptides interact could have a specific regulatory function. To this end, we compared the transcriptional activity of wtERα with that of the ER-L372R mutant in a reconstituted transcriptional system in transfected cells. Specifically, the luciferase reporter plasmid 3×ERE-TATA-Luc and increasing concentrations of wild-type or mutant ERα expression vector were transiently transfected into ER-negative HepG2 cells. All transfections were performed in the absence or presence of 10^{-7} M 17β-estradiol, 4-hydroxytamoxifen, or ICI 182,780. As shown in Fig. 4A, the constitutive transcriptional activity of ER-L372R was considerably lower than that displayed by the wild-type receptor. This effect is probably a general consequence of disturbances of the ER-LBD surface, as other mutations in ERα helix 12 analyzed in the same manner also exhibit a lower level of constitutive activity (data not shown). Tamoxifen manifests significant partial agonist activity in HepG2 cells expressing exogenous wtERα, the magnitude of which is increased by a maximal 3-fold when assayed in

EXHIBIT F

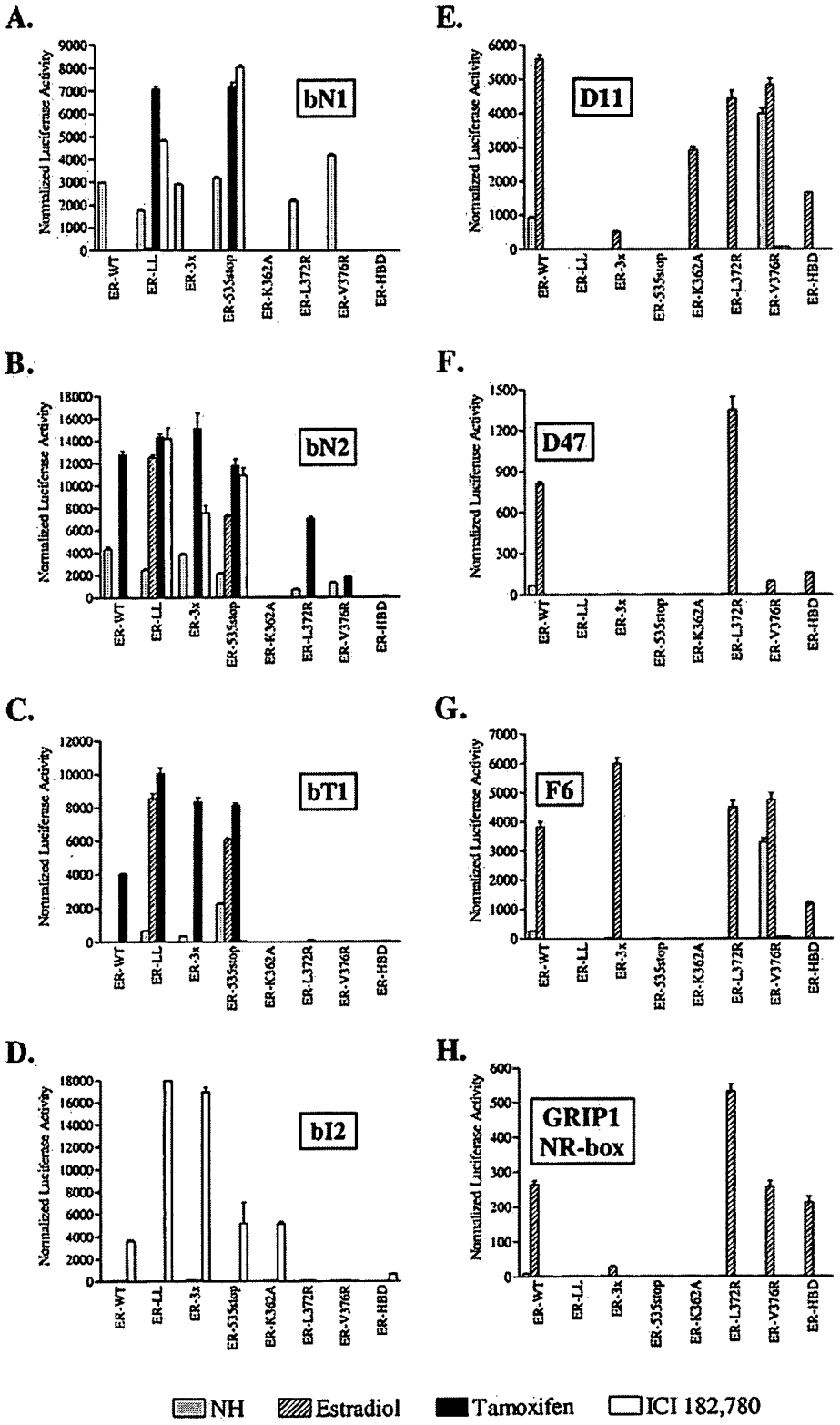


Fig. 3. The Binding Surface for CoRNR Box-Containing Peptides Is Located on Helixes H3 and H5, But Not H12, of ERα

Mammalian two-hybrid assays were performed using wild-type or mutant VP16-ERα and Gal4-DBD-CoRNR box or Gal4-DBD-LXXLL fusion peptides. The indicated vectors were transfected into HepG2 cells with a reporter plasmid (5×Gal4-TATA-Luc) and the control β-galactosidase plasmid. After transfection, cells were treated with 100 nM hormone for 24 h before being harvested for determination of luciferase and β-galactosidase activities. Data are presented as normalized luciferase activity, which was obtained by dividing luciferase activity by β-galactosidase activity.

EXHIBIT F

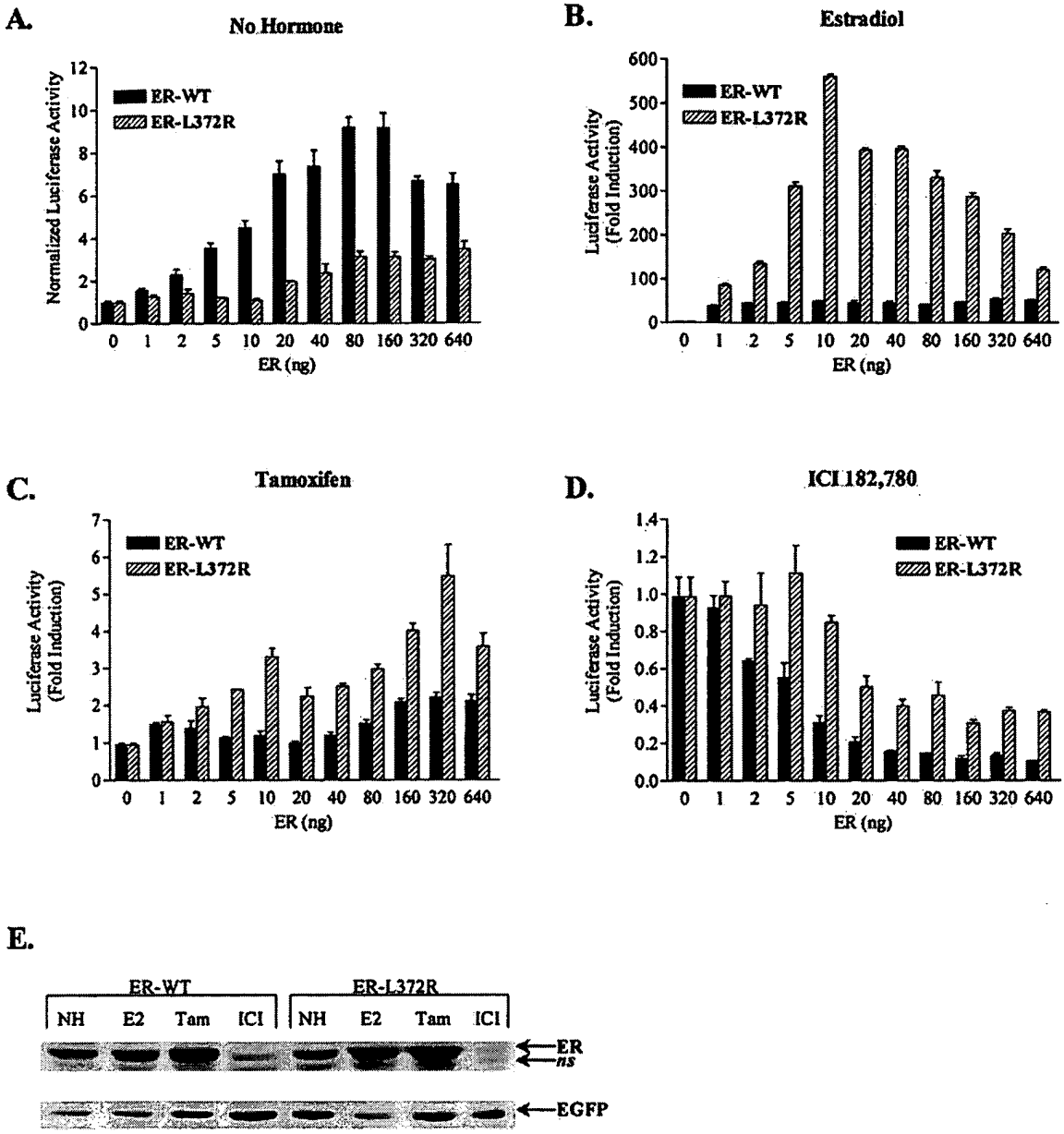


Fig. 4. Analysis of the Transcriptional Activity of Mutant ERα with Amino Acid Substitution at Leucine 372

A–D, HepG2 cells were transfected with different amounts of wild-type or mutant ERα along with 3×ERE-TATA-Luc reporter and the β-galactosidase control plasmid. After transfection, cells were treated with different ligands (100 nM) for 24 h before being harvested for determination of luciferase and β-galactosidase activities. Data in A are presented as normalized luciferase activity, which was obtained by dividing luciferase activity by β-galactosidase activity. Data in B, C, and D are presented as fold induction, which was obtained by dividing the normalized luciferase activity in the presence of ligand by that in the absence of ligand. E, Western analysis of the expression levels of wild-type or mutant ERα. HepG2 cells were cotransfected with wild-type or mutant ERα and a control green fluorescence protein expression vector. After 24 h of hormone treatment, nuclear extracts were prepared from transfected HepG2 cells and analyzed as described in *Materials and Methods*.

cells expressing the L372R mutant (Fig. 4C). In this cell system the pure antiestrogen ICI 182,780 functions as an inverse agonist, and in the absence of added agonist it caused a 90% repression of the basal transcriptional activity of the 3×ERE-TATA-Luc reporter in cells expressing wtERα. This repression activity is decreased by a maximal 4-fold when a similar assay is performed in cells expressing the ER-L372R mutant (Fig. 4D). The most dramatic and unexpected result observed, however, was that estradiol-stimulated transcriptional activity of the ER-L372R mutant was greatly enhanced compared with that of wtER (Fig. 4B). This observed increase in the efficacy of estradiol was most dramatic when 10 ng of the ER-L372R expression vector was used (11-fold more active than wtER). Transfection of higher

EXHIBIT F

concentrations of ER-L372 led to a progressive decrease in estradiol-dependent transcriptional activity, possibly due to sequestration of limiting transcription factors by overexpression of this highly active mutant receptor. Similar results were observed using a C3-Luc reporter that contains a *bona fide* estrogen-responsive human C3 complement promoter (data not shown). Western analysis indicated that the wild-type and mutant ERs used in this study were similarly expressed, ruling out differences in expression level as the basis for the observed differences in transcriptional activity (Fig. 4E). These results suggest that amino acid residue L372 is contained within a region that functions as a negative regulatory surface for ERα.

The ER-L372R Mutant Is Not Hypersensitive to Estradiol

The dramatically enhanced estradiol-dependent transcriptional activity of ER-L372R prompted us to examine whether this mutant receptor displayed an increased sensitivity to estradiol. To address this issue, HepG2 cells were transfected with 10 ng of either the wtERα or ER-L372R expression vector along with 3×ERE-TATA-Luc reporter and subsequently treated with increasing concentrations of estradiol (Fig. 5). Contrary to what was expected, we observed that the mutant receptor was about one order of magnitude less sensitive to estradiol than the wild-type receptor. These results suggest that mutation at leucine 372 of ERα did not render the receptor more sensitive to estradiol treatment.

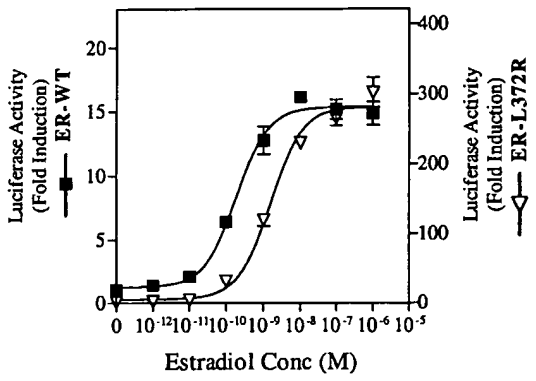


Fig. 5. Dose-Response Analysis of 3×ERE-TATA-Luc Induction by Estradiol for the wtER or Mutant ER-L372R

HepG2 cells were transfected with 10 ng ER-WT or ER-L372R along with 3×ERE-TATA-Luc and the β-galactosidase control plasmid. After transfection, cells were treated with increasing concentrations of estradiol for 24 h before being harvested for determinations of luciferase and β-galactosidase activities. Data are presented as fold induction, which was obtained by dividing the normalized luciferase activity in the presence of ligand by that in the absence of ligand. Note that the scale for ER-WT (*left* y-axis) is about 1/20th that for ER-L372R (*right* y-axis).

Increased Interaction with Known LXXLL Motif-Containing Peptides May Partly Account for the Enhanced Transcriptional Activity of the ER-L372R Mutant

One mechanism by which the L372R mutation could exhibit higher estradiol-mediated transcriptional activity is through an increased binding affinity or binding capacity for coactivators. The abilities of wtERα and ER-L372R to interact with different classes of LXXLL-containing peptides were compared in the presence of increasing concentrations of estradiol using a mammalian two-hybrid assay (Fig. 6). The Gal4-DBD-D11, Gal4-DBD-D47, Gal4-DBD-F6, and Gal4-DBD-GRIP1 NR-box constructs were described above. The Gal4-DBD-SRC-1 NR-box fusion contains the middle three copies of the LXXLL motif found in the coactivator SRC-1. As shown in Fig. 6, compared with the wtERα, the ER-L372R mutant did not show a higher affinity for any of the LXXLL peptides tested. However, ER-L372R did exhibit a higher capacity for binding to the GRIP1 NR-box peptide at higher concentrations of estradiol (Fig. 6D). Therefore, the enhanced transcriptional activity of ER-L372R can be explained in part by increased coactivaor binding.

ER-Interacting CoRNR Box Peptides Show Distinct Preferences for Other Nuclear Receptors

It has been shown that different members of the nuclear receptor superfamily display distinct preferences for the known IDs located within NCoR and SMRT (21). Consequently, we examined the binding specificity of the ER-interacting CoRNR box peptides with other nuclear receptors using the mammalian two-hybrid assay. Surprisingly, although these peptides were designed using the CoRNR box consensus derived from NCoR and SMRT, which interacts with both apo-RARα and apo-TRβ, none of the CoRNR box-containing peptides obtained in this study interacted with these two receptors (see Fig. 1, C and D). However, we noticed that the bN2 peptide can interact with apo-RXRα, and treatment with 9-*cis*-retinoic acid abolished this interaction (Fig. 7A). Under the same conditions apo-RXR showed only minimal binding to NCoR/SMRT IDs, and the interaction between SMRT ID-C and apo-RXR was actually slightly increased, rather than inhibited, by retinoic acid treatment (Fig. 7A), consistent with previous observations that RXRα only weakly interacts with SMRT, and retinoic acid treatment can strengthen this interaction (21, 24). The binding of progesterone receptor (PR) to ER-interacting CoRNR box peptides was also examined. Figure 7, B and C, showed that no interaction of PRA and PRB with the CoRNR box peptides was observed in the absence of hormone or in the presence of progesterone. However, treatment with RU486 (a mixed agonist/antagonist) or ZK98299 (a pure antagonist) facilitated a robust interaction between both PR isoforms and the bT1 peptide. As antagonist-bound PRs can interact

EXHIBIT F

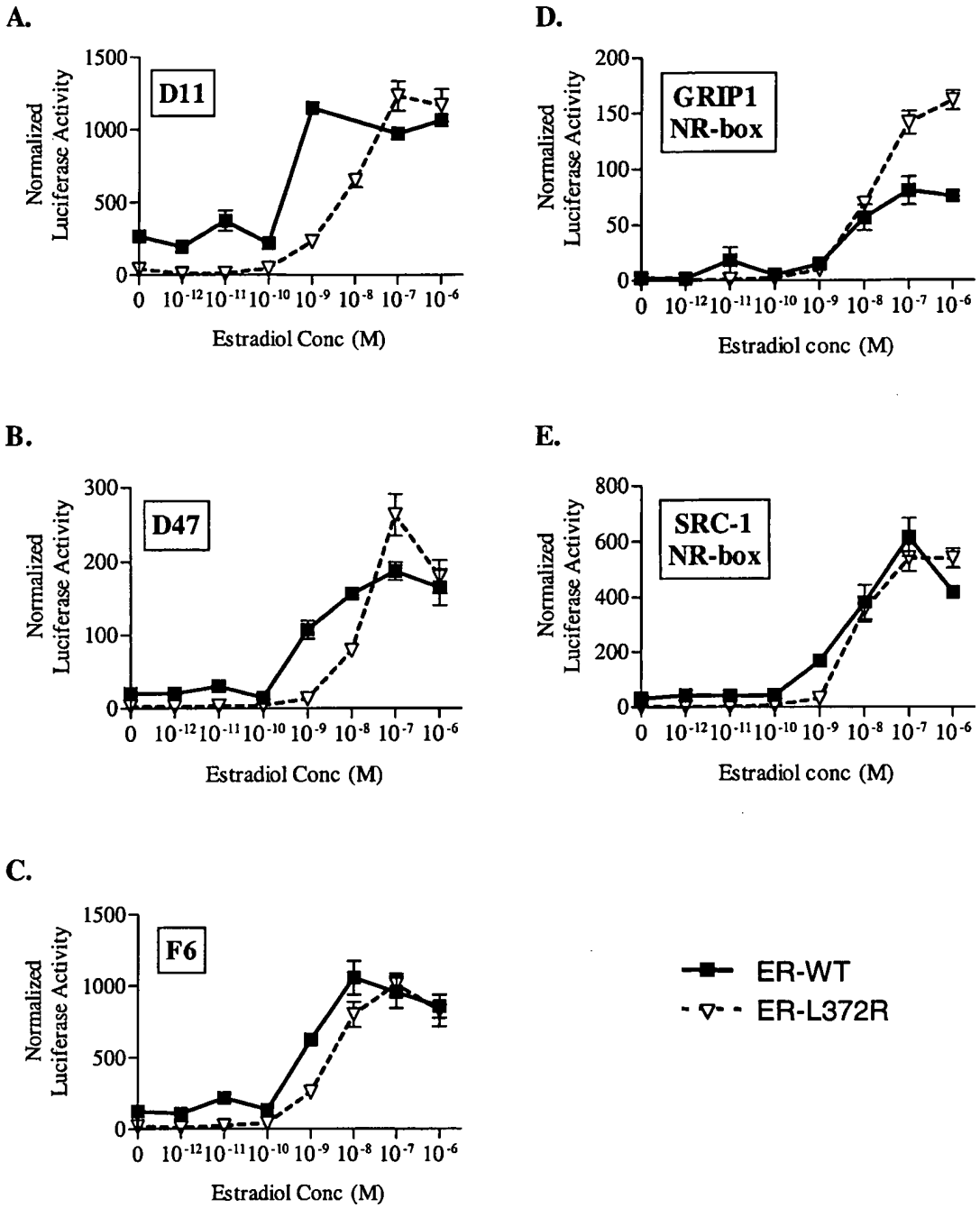


Fig. 6. ER-L372R Shows Higher Capacity for Binding to LXXLL Motif-Containing Peptides

VP16-ER-WT or VP16-ER-L372R was transfected into HepG2 cells along with 3×ERE-TATA-Luc, the β-galactosidase control plasmid, and different LXXLL motif-containing peptides as indicated in each panel. After transfection, cells were treated with increasing concentrations of estradiol for 24 h before being harvested for determination of luciferase and β-galactosidase activities. Data are presented as normalized luciferase activity, which was obtained by dividing luciferase activity by β-galactosidase activity.

with NCoR/SMRT IDs, as shown in Fig. 7, B and C, and in previous studies (25), it is not surprising that CoRNR box-containing peptides with this binding specificity were found in our screens. However, there are differences in the CoRNR box binding patterns of PR treated with RU486 or ZK98299. The bT1 peptide, which binds tamoxifen-bound ERα and ERβ, associates more strongly with PRs treated with RU486 than ZK98299, whereas NCoR/SMRT ID-Cs exhibit a stronger interaction with ZK98299-bound PRs than RU486-bound PRs. These results demonstrated that the flanking sequences of the CoRNR box can dictate the

EXHIBIT F

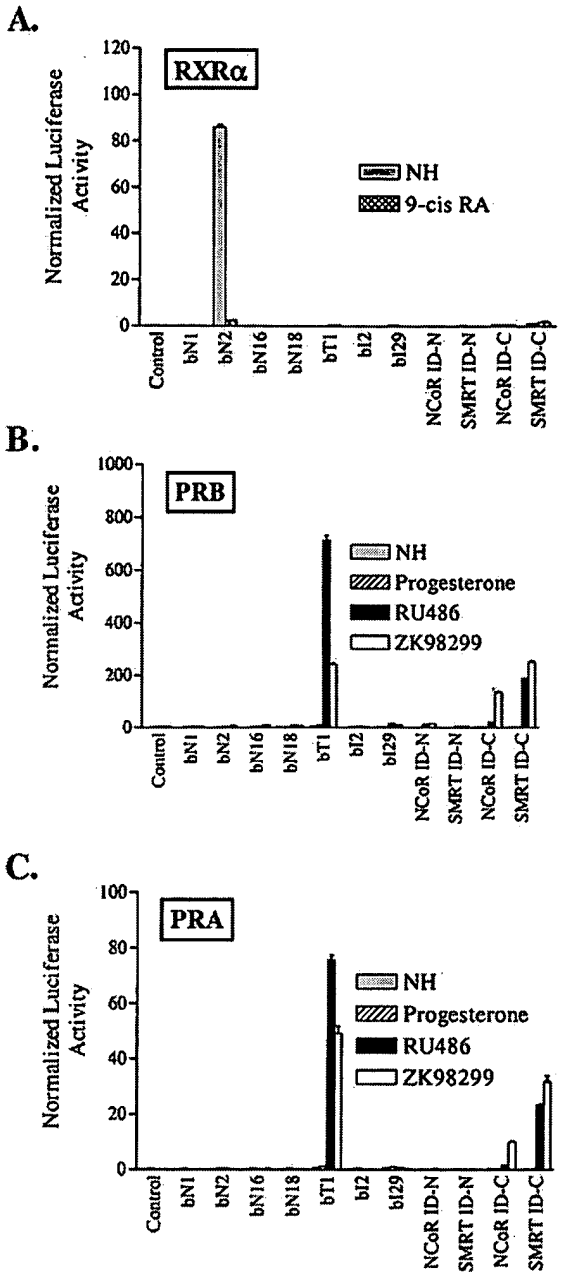


Fig. 7. ER-Interacting CoRNR Box Peptides Show Distinct Preferences for Other Nuclear Receptors

The ability of the CoRNR box-containing peptides to interact with RXRα (A), PRB (B), or PRA (C) was tested using mammalian two-hybrid assays. HepG2 cells were transfected with VP16-receptor fusion expression vector along with the Gal4-DBD-peptide fusion construct, the 5×Gal4-TATA-Luc reporter, and the β-galactosidase control plasmid. After transfection, cells were treated with 100 nM hormone for 24 h before being harvested for determination of luciferase activity. Data are presented as normalized luciferase activity, which was obtained by dividing luciferase activity by β-galactosidase activity.

receptor binding specificity and that an unidentified corepressor(s) other than NCoR/SMRT might exist that shares the CoRNR box motif, but differentially interacts with different nuclear receptors.

DISCUSSION

It is now generally accepted that the transcriptional activity of nuclear receptors is regulated by their ability to couple with appropriate coactivator or corepressor proteins and by the relative and absolute levels of these two classes of receptor-associated proteins in target cells. Whereas the nature of the interaction of receptors with coactivators is generally understood, there is little known about the receptor-corepressor interface. Initial studies indicated that corepressor (NCoR/SMRT) binding requires an intact CoR box conserved in helix 1 of the unliganded TRβ and RXRα (26, 27). The generality of the requirement of helix 1 for corepressor binding, however, is unclear, because this domain is not highly conserved in ERα. It now appears more likely that the CoR box contributes to the overall structure of a distal corepressor binding site on TR, but does not provide the primary point of contact between the receptor and corepressors (28). This conclusion is further supported by the recent crystallographic analysis of peroxisome proliferator-activated receptor α (PPARα) complexed with antagonist GW6471 and SMRT ID-C, which reveals that the primary corepressor binding pocket overlaps that required for coactivator binding in helixes 3, 4, and 5 (29). No evidence for a contribution of sequences in helix 1 is apparent from this structure. Indeed, before the solution of this informative crystal structure the involvement of helixes 3 and 5 within TRα and RXRα in corepressor binding was implicated by mutagenesis (20). In the present study mutations of these corresponding amino acid residues in ERα were made, and the resultant mutants were tested for their ability to interact with a series of CoRNR box- and LXXLL motif-containing peptides. Two of the ERα mutants, ER-K362A and ER-V376R, exhibited impaired interactions with both LXXLL- and CoRNR box-containing peptides, confirming that the surfaces required for coactivator and corepressor binding overlap. The most informative mutation, however, is ER-L372R, which retained the LXXLL peptide binding characteristics of wtER, but was unable to interact with two of the CoRNR box peptides (bT1 and bI2) identified in our studies. Using this mutant we were able to dissociate corepressor and coactivator binding by demonstrating that the agonist efficacy of tamoxifen and estradiol could be dramatically enhanced when the binding site for these CoRNR box peptides was disrupted. The conclusion that ER-L372 was required for corepressor binding was supported by the additional observations that the inverse agonist activity of the pure antiestrogen ICI 182,780 was decreased when assayed on this mutant. Cumulatively, these findings provide strong evidence for the involvement of corepressors in ER action.

It has been shown that the corepressors NCoR and SMRT can associate with ERα in the presence of tamoxifen (13, 16, 30, 31). However, in the current

EXHIBIT F

study we were unable to detect an interaction between ER and the CoRNR box peptides, which contain the NCoR/SMRT IDs, although these peptides were capable of binding apo-TRβ and apo-RARα. It is possible that the interaction interface between ER and NCoR/SMRT lies outside of the CoRNR box motif. However, identification of CoRNR box peptides that bind ER in the presence of antagonists also suggests that there exist other CoRNR box-containing proteins that may be involved in ER signaling.

There is strong evidence that corepressors NCoR and SMRT can modulate the agonist activity of tamoxifen, but they play little, if any, role in regulating the physiological actions of estradiol (14, 17). Predictably, we observed that the agonist activity of tamoxifen was enhanced by disruption of the putative corepressor binding surface (defined by the L372R mutation) on ERα. However, one of the most interesting findings of our study was that this mutation also enhanced the agonist efficacy of estradiol. One possible explanation is that this mutation disrupts the interaction of ERα with a corepressor (or a competitive repressor) that, unlike NCoR and SMRT, can recognize the agonist-activated structure of ERα. Potential candidates that could function in this manner include repressor of estrogen receptor activity (REA), short heterodimer partner (SHP), and dosage-sensitive sex reversal-adrenal hypoplasia congenita critical region on the X chromosome (DAX-1) (32–34). We believe that the data are also consistent with the hypothesis that there exist corepressors that can interact with apo-ERα or ERα bound to an antagonist and that the L372R mutation disrupts this interaction and in doing so reduces the energy of activation of the receptor by removing the "brake" afforded by the corepressor. This hypothesis will be tested in future studies.

It is interesting to note that the cocrystal structure of ERα with NR box 2 of GRIP1 predicts that K362, L372, and V376 make van der Waals contacts with the leucine side-chains within the LXXLL motif, and that K362 can also form hydrogen bonds with the backbone of this peptide (8). Although the transcriptional activity of the ER-V376R and ER-K362A mutants was compromised (data not shown), we were surprised to find that mutation of amino acid leucine 372 to arginine within ERα did not decrease, but greatly enhanced, estradiol efficacy. Moreover, ER-L372R retained the ability to interact with all three classes of LXXLL motif-containing peptides and actually showed an increased binding capacity for the GRIP1 NR box peptides. It is likely that L372 of ERα, which corresponds to L302 in PPARα, plays a significant role in interacting with CoRNR box-containing corepressors, as suggested by the recent crystal structure of the antagonist-PPARα bound to the SMRT ID-C (29). Alternatively, L372 could be important for mediating the autoinhibitory activity of ERα through an intramolecular interaction. Whatever the mechanism, mutation at L372 disrupts the negative regulatory surface on ER, allowing the receptor to be more susceptible to agonist activation. Another possible explanation is that mutation of L372 to an arginine may inadvertently increase the affinity of the receptor for coactivators by making additional contacts with the amino acid residues adjacent to the LXXLL motif. Given the overlapping nature of the corepressor and coactivator binding site on ERα, it is likely that the increased transcriptional activity of ER-L372R is due to both a loss of corepressor binding and an increase in the ability of coactivators to bind to the receptor.

Inspection of the crystal structure of human ERα LBD in the presence of estradiol indicated that amino acid residues in helixes 3, 4, 5, and 12 can form a hydrophobic surface that constitutes the primary contact point for the LXXLL motif found in the p160 coactivators (8). However, helix 12 is not required for corepressor binding, as its deletion or mutation only serves to enhance the interaction of both NCoR and SMRT with nuclear receptors (27, 28, 35). One of the most interesting observations of the current study was that the binding of the CoRNR box peptides to ERα was not negatively affected by mutations in helix 12. In fact, most of these CoRNR box peptides interacted better with ERα mutants in which the helix 12 structure was compromised, demonstrating a negative regulatory function of helix 12 for corepresssor binding. In addition, it was found that the exquisite ligand specificity demonstrated by the different classes of CoRNR box peptides identified was lost when helix 12 was disrupted. Consistent with these results, a recent report demonstrated that a mutant ERα deleted for the C-terminal 58 amino acids can be coimmunoprecipitated with SMRT in the absence of hormone or in the presence of estradiol and tamoxifen, whereas under the same conditions wtERα can only interact with SMRT in a tamoxifen-dependent manner (36). Thus, although ERα helix 12 is not required for corepressor binding, its relative positioning by different agonists/antagonists can regulate the interactions between corepressors and the receptor.

In summary, using combinatorial phage display, three different classes of CoRNR box-containing peptides have been identified that bind selectively to apo-ER, tamoxifen-bound ER, and ICI 182,780-bound ER. These peptides have helped map a negative regulatory surface within ERα. Our findings are consistent with the existence of corepressors that interact with and modulate ERα activity. Although NCoR/SMRT may be important for mediating the tamoxifen antagonist activity of ERα, we cannot rule out the possibility that additional corepressors may be involved in regulating ERα activity in the presence of antagonists/agonists.

MATERIALS AND METHODS

Chemicals

17β-Estradiol, 4-hydroxytamoxifen, T_3, all-*trans*-retinoic acid, 9-*cis*-retinoic acid, and progesterone were obtained from

EXHIBIT F

Sigma (St. Louis, MO). ICI 182,780 was obtained from Tocris (Ellisville, MO). RU486 was a gift from Ligand Pharmaceuticals, Inc. (San Diego, CA). ZK98299 was a gift from Schering AG (Berlin, Germany).

Construction of the Phage Display Library

A focused CoRNR box phage display library was constructed using a degenerate oligonucleotide able to code for 23 amino acids in the format of X_7-L-X-X-H/I-I-X-X-X-I/L-X_7, where L is leucine, H is histidine, I is isoleucine, and X is any amino acid. A 100-bp single-stranded oligonucleotide containing terminal *Xho*I and *Xba*I sites and fully degenerate at the remaining (NNK) positions was synthesized, converted to double-stranded form, and purified by gel electrophoresis. The restricted oligonucleotides were ligated into the mBAX vector, and the ligated DNA was used to transform *Escherichia coli* DH5αF′ competent cells. The transformants were harvested, and the phage were purified from the transformants. The library has a complexity of 1.3×10^7 different peptide sequences.

Panning of CoRNR Box Peptide Library

Enrichment and amplification of the library using a target protein were carried out as described previously with minor modifications (23). To isolate phage that bind tamoxifen- or ICI 182,780-bound ER, 4 pmol ERα or ERβ were diluted in $NaHCO_3$ plus 10^{-6} M antiestrogen and adsorbed onto an Immulon 4 plate (Thermo Labsystems, Helsinki, Finland) for 3 h at room temperature. To isolate apo-ER binding phage, Immulon 4 plates were coated with streptavidin in 0.1 M $NaHCO_3$ and then incubated for 1 h with 2 pmol biotinylated vitellogenin ERE, followed by incubation for 1 h with 4 pmol ERα or ERβ. After blocking the ER-coated or ER/ERE-coated plates for 1 h with 0.1% BSA in $NaHCO_3$, aliquots of the phage library (10^{10} plaque-forming units) were diluted in PBS containing 0.1% Tween 20, and phage were allowed to bind overnight at 4 C. Unbound phage were subsequently removed by washing with PBS containing 0.1% Tween 20, and bound phage were eluted with 50 mM glycine-HCl (pH 2.0), followed by 100 mM ethanolamine (pH 11.0). The pH of the first eluate was immediately neutralized with 200 mM Na_2HPO_4 (pH 8.5) and combined with the first eluate. Eluted phage were amplified in DH5αF′ cells for 5 h, and the supernatant containing amplified phage was collected for use in subsequent rounds of panning. After the third round of panning, randomly selected phage plaques were amplified, and their single-stranded DNA was sequenced. Phage amplified from single plaques and purified were tested for binding to ERα or ERβ with or without hormones using ELISA, as described previously (23).

Cell Culture and Transient Transfection

HepG2 (hepatocellular carcinoma) and HeLa (cervical carcinoma) cells were maintained in MEM (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone Laboratories, Inc., Logan, UT), 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (Invitrogen) and maintained in a humidified 37 C incubator with 5% CO_2. Cells were plated into 24-well plates and transfected using lipofectin reagent according to the manufacturer's instructions (Invitrogen). A transfection mixture containing a total of 3000 ng plasmid in each of triplicate samples was incubated with cells for 3 h (for HeLa) or 5–16 h (for HepG2). Hormones were added to the cells 24 h before harvest. Luciferase and β-galactosidase activities were measured as previously described (37). For mammalian two-hybrid assays, 1500 ng 5×Gal4-TATA-Luc reporter plasmid, 500 ng VP16-receptor fusion, 500 ng Gal4-DBD-peptide fusion, and 500 ng normalization plasmid pCMVβgal were used. For receptor transcriptional activity assays, 1500 ng 3×ERE-TATA-Luc, 100 ng pCMVβgal, and 0–640 ng wild-type or mutant pRST7-ERα were used. A pBluescript II vector (Stratagene, La Jolla, CA) was added in these experiments to balance the amount of input DNA in transfections.

Plasmids

The Gal4-DBD-peptide fusions were constructed by excising DNA sequences coding for the peptides from mBAX vector with *Xho*I and *Xba*I and subcloning it into the *Sal*I and *Xba*I sites of the pMsx vector (23). To generate the Gal4-DBD-NCoR ID-N, Gal4-DBD-SMRT ID-N, Gal4-DBD-NCoR ID-C, or Gal4-DBD-SMRT ID-C fusions, single-stranded oligonucleotides containing the DNA sequences coding for the individual ID and terminal *Xho*I and *Xba*I sites were synthesized and converted to double-stranded form, and the restricted oligonucleotides were ligated into the pMsx vector. Gal4-DBD-D11, Gal4-DBD-D47, Gal4-DBD-F6, Gal4-DBD-GRIP1 NR-box, and Gal4-DBD-SRC-1 NR-box were described previously (23). Mammalian expression plasmid pRST7-ERα has been described previously (38). Mutant receptors pRST7-ER-K362A, pRST7-ER-L372R, and pRST7-ER-V376R were generated by using the QuikChange sitedirected mutagenesis kit (Stratagene), with wild-type pRST7-ERα as the template. The reporter 3×ERE-TATA-Luc has been described previously (22). Construction of VP16-ERα, VP16-ERβ, VP16-ER-LL, VP16-ER-3×, VP16-ER-535 stop, VP16-RARα, and VP16-RXRα were described previously (23). VP16-TRβ was provided by D. D. Moore (Baylor College of Medicine, Houston, TX). VP16-PRA and VP16-PRB were gifts from C. X. Wen (Ligand Pharmaceuticals, Inc.). VP16-ER-HBD was generated by PCR of the full-length human ERα cDNA with primers containing *Eco*RI and *Sal*I sites and subsequently subcloning the PCR product into the *Eco*RI and *Sal*I sites in the pVP16 vector (CLONTECH Laboratories, Inc., Palo Alto, CA). Other VP16-ERα point mutants (ER-K362A, ER-L372R, and ER-V376R) were constructed by excision of mutant ER cDNAs from their corresponding mammalian expression plasmids and subcloned into the pVP16 vector. The reporter 5×Gal4-TATA-Luc was a gift from X. F. Wang (Duke University Medical Center, Durham, NC).

Western Blot Analysis

HepG2 cells were plated into 100-mm plates and transfected with 3 μg of the different forms of receptor treated with different hormones together with 1 μg of a green fluorescent protein expression vector for normalization. Nuclear extracts were prepared as described previously (39). Proteins (20-μg samples) were separated on sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose. The receptors were detected with the monoclonal antibody H222 (provided by Geoffrey Greene, Ben May Institute, Chicago, IL). Green fluorescent protein was probed with an anti-green fluorescent protein polyclonal antibody (CLONTECH Laboratories, Inc.). The immunocomplexes were visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ) as described by the manufacturer.

Acknowledgments

The authors thank C.-Y. Chang for providing the VP16-ER-HBD plasmid.

Received March 5, 2002. Accepted April 18, 2002.

Address all correspondence and requests for reprints to: Dr. Donald P. McDonnell, Department of Pharmacology and

EXHIBIT F

Cancer Biology, Duke University Medical Center, Box 3813, Durham, North Carolina 27710. E-mail: donald.mcdonnell@duke.edu.

This work was supported by NIH Grants DK-48807 and CA-90645.

REFERENCES

1. Chambraud B, Berry M, Redeuilh G, Chambon P, Baulieu EE 1990 Several regions of human estrogen receptor are involved in the formation of receptor-heat shock protein 90 complexes. J Biol Chem 265:20686–20691
2. Pratt WB, Toft DO 1997 Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev 18:306–360
3. Lee HS, Aumais J, White JH 1996 Hormone-dependent transactivation by estrogen receptor chimeras that do not interact with hsp90. Evidence for transcriptional repressors. J Biol Chem 271:25727–25730
4. Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson JA 1996 Cloning of a novel receptor expressed in rat prostate and ovary. Proc Natl Acad Sci USA 93:5925–5930
5. Pettersson K, Grandien K, Kuiper GG, Gustafsson JA 1997 Mouse estrogen receptor β forms estrogen response element-binding heterodimers with estrogen receptor α. Mol Endocrinol 11:1486–1496
6. Hall JM, McDonnell DP 1999 The estrogen receptor β-isoform (ERβ) of the human estrogen receptor modulates ERα transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. Endocrinology 140:5566–5578
7. Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA, Carlquist M 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. Nature 389:753–758
8. Shiau AK, Barstad D, Loria PM, Cheng L, Kushner PJ, Agard DA, Greene GL 1998 The structural basis of estrogen receptor/coactivator recognition and the antagonism of this interaction by tamoxifen. Cell 95:927–937
9. Pike AC, Brzozowski AM, Walton J, Hubbard RE, Thorsell AG, Li YL, Gustafsson JA, Carlquist M 2001 Structural insights into the mode of action of a pure antiestrogen. Structure 9:145–153
10. McDonnell DP, Clemm DL, Hermann T, Goldman ME, Pike JW 1995 Analysis of estrogen receptor function *in vitro* reveals three distinct classes of antiestrogens. Mol Endocrinol 9:659–669
11. Paige LA, Christensen DJ, Gron H, Norris JD, Gottlin EB, Padilla KM, Chang CY, Ballas LM, Hamilton PT, McDonnell DP, Fowlkes DM 1999 Estrogen receptor (ER) modulators each induce distinct conformational changes in ERα and ERβ. Proc Natl Acad Sci USA 96:3999–4004
12. Wijayaratne AL, Nagel SC, Paige LA, Christensen DJ, Norris JD, Fowlkes DM, McDonnell DP 1999 Comparative analyses of mechanistic differences among antiestrogens. Endocrinology 140:5828–5840
13. Lavinsky RM, Jepsen K, Heinzel T, Torchia J, Mullen TM, Schiff R, Del-Rio AL, Ricote M, Ngo S, Gemsch J, Hilsenbeck SG, Osborne CK, Glass CK, Rosenfeld MG, Rose DW 1998 Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. Proc Natl Acad Sci USA 95:2920–2925
14. Smith CL, Nawaz Z, O'Malley BW 1997 Coactivator and corepressor regulation of the agonist/antagonist activity of the mixed antiestrogen, 4-hydroxytamoxifen. Mol Endocrinol 11:657–666
15. McKenna NJ, O'Malley BW 2000 An issue of tissues: divining the split personalities of selective estrogen receptor modulators. Nat Med 6:960–962
16. Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M 2000 Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 103:843–852
17. Jepsen K, Hermanson O, Onami TM, Gleiberman AS, Lunyak V, McEvilly RJ, Kurokawa R, Kumar V, Liu F, Seto E, Hedrick SM, Mandel G, Glass CK, Rose DW, Rosenfeld MG 2000 Combinatorial roles of the nuclear receptor corepressor in transcription and development. Cell 102: 753–763
18. Zhang X, Jeyakumar M, Petukhov S, Bagchi MK 1998 A nuclear receptor corepressor modulates transcriptional activity of antagonist-occupied steroid hormone receptor. Mol Endocrinol 12:513–524
19. Perissi V, Staszewski LM, McInerney EM, Kurokawa R, Krones A, Rose DW, Lambert MH, Milburn MV, Glass CK, Rosenfeld MG 1999 Molecular determinants of nuclear receptor-corepressor interaction. Genes Dev 13: 3198–3208
20. Hu X, Lazar MA 1999 The CoRNR motif controls the recruitment of corepressors by nuclear hormone receptors. Nature 402:93–96
21. Wong CW, Privalsky ML 1998 Transcriptional silencing is defined by isoform- and heterodimer-specific interactions between nuclear hormone receptors and corepressors. Mol Cell Biol 18:5724–5733
22. Norris JD, Fan D, Stallcup MR, McDonnell DP 1998 Enhancement of estrogen receptor transcriptional activity by the coactivator GRIP-1 highlights the role of activation function 2 in determining estrogen receptor pharmacology. J Biol Chem 273:6679–6688
23. Chang Cy, Norris JD, Gron H, Paige LA, Hamilton PT, Kenan DJ, Fowlkes D, McDonnell DP 1999 Dissection of the LXXLL nuclear receptor-coactivator interaction motif using combinatorial peptide libraries: discovery of peptide antagonists of estrogen receptors α and β. Mol Cell Biol 19:8226–8239
24. Chen JD, Evans RM 1995 A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature 377:454–457
25. Wagner BL, Norris JD, Knotts TA, Weigel NL, McDonnell DP 1998 The nuclear corepressors NCoR and SMRT are key regulators of both ligand- and 8-bromo-cyclic AMP-dependent transcriptional activity of the human progesterone receptor. Mol Cell Biol 18:1369–1378
26. Horlein AJ, Naar AM, Heinzel T, Torchia J, Gloss B, Kurokawa R, Ryan A, Kamei Y, Soderstrom M, Glass CK, et al. 1995 Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature 377:397–404
27. Zhang J, Hu X, Lazar MA 1999 A novel role for helix 12 of retinoid × receptor in regulating repression. Mol Cell Biol 19:6448–6457
28. Marimuthu A, Feng W, Tagami T, Nguyen H, Jameson JL, Fletterick RJ, Baxter JD, West BL 2002 TR Surfaces and conformations required to bind nuclear receptor corepressor. Mol Endocrinol 16:271–286
29. Xu HE, Stanley TB, Montana VG, Lambert MH, Shearer BG, Cobb JE, McKee DD, Galardi CM, Plunket KD, Nolte RT, Parks DJ, Moore JT, Kliewer SA, Willson TM, Stimmel JB 2002 Structural basis for antagonist-mediated recruitment of nuclear co-repressors by PPAR. Nature 415:813–817
30. Yamamoto Y, Wada O, Suzawa M, Yogiashi Y, Yano T, Kato S, Yanagisawa J 2001 The tamoxifen-responsive estrogen receptor α mutant D351Y shows reduced tamoxifen-dependent interaction with corepressor complexes. J Biol Chem 276:42684–42691
31. Shang Y, Brown M 2002 Molecular determinants for the tissue specificity of SERMs. Science 295:2465–2468
32. Delage-Mourroux R, Martini PG, Choi I, Kraichely DM, Hoeksema J, Katzenellenbogen BS 2000 Analysis of estrogen receptor interaction with a repressor of estrogen

EXHIBIT F

receptor activity (REA) and the regulation of estrogen receptor transcriptional activity by REA. J Biol Chem 275:35848–35856
33. Johansson L, Thomsen JS, Damdimopoulos AE, Spyrou G, Gustafsson JA, Treuter E 1999 The orphan nuclear receptor SHP inhibits agonist-dependent transcriptional activity of estrogen receptors ERα and ERβ. J Biol Chem 274:345–353
34. Zhang H, Thomsen JS, Johansson L, Gustafsson JA, Treuter E 2000 DAX-1 functions as an LXXLL-containing corepressor for activated estrogen receptors. J Biol Chem 275:39855–39859
35. Gurnell M, Wentworth JM, Agostini M, Adams M, Collingwood TN, Provenzano C, Browne PO, Rajanayagam O, Burris TP, Schwabe JW, Lazar MA, Chatterjee VK 2000 A dominant-negative peroxisome proliferator-activated receptor γ (PPARγ) mutant is a constitutive repressor and inhibits PPARγ-mediated adipogenesis. J Biol Chem 275:5754–5759
36. Jung DJ, Lee SK, Lee JW 2001 Agonist-dependent repression mediated by mutant estrogen receptor α that lacks the activation function 2 core domain. J Biol Chem 276:37280–37283
37. Norris J, Fan D, Aleman C, Marks JR, Futreal PA, Wiseman RW, Iglehart JD, Deininger PL, McDonnell DP 1995 Identification of a new subclass of Alu DNA repeats which can function as estrogen receptor-dependent transcriptional enhancers. J Biol Chem 270: 22777–22782
38. Dana SL, Hoener PA, Wheeler DA, Lawrence CB, McDonnell DP 1994 Novel estrogen response elements identified by genetic selection in yeast are differentially responsive to estrogens and antiestrogens in mammalian cells. Mol Endocrinol 8:1193–1207
39. Schreiber E, Matthias P, Muller MM, Schaffner W 1989 Rapid detection of octamer binding proteins with 'mini-extracts,' prepared from a small number of cells. Nucleic Acids Res 17:6419